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The regulation of post-germinative transition from the cotyledon- to vegetative-leaf stages by microRNA-targeted *SQUAMOSA PROMOTER-BINDING PROTEIN LIKE13* in *Arabidopsis*

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Abstract

Germination and early seedling development are critical for successful stand establishment of plants. Following germination, the cotyledons, which are derived from embryonic tissue, emerge from the seed. *Arabidopsis* seedlings at post-germinative stages are supported mainly by the supply of nutrition from the cotyledons until vegetative leaves emerge and initiate photosynthesis. The switch to autotrophic growth is a significant transition at the post-germinative stage. Here, we provide evidence that down-regulation of *SQUAMOSA PROMOTER-BINDING PROTEIN LIKE13* (*SPL13*) by microRNA156 (miR156) plays an important role in the regulation of the post-germinative switch from the cotyledon stage to the vegetative-leaf stage. Silent mutations created in the *SPL13* sequence in the region that is complementary to the miR156 sequence caused the deregulation of the mutant form of *SPL13* (*mSPL13*) mRNA from miR156. Mutant seedlings over-accumulated miRNA-resistant messages and exhibited a delay in the emergence of vegetative leaves compared to wild-type seedlings. The delay was not observed in control transgenic plants expressing non-mutated *SPL13*, indicating that the phenotype was caused specifically by the silent mutations and deregulation of *SPL13* from miR156. Characterization of the *SPL13* promoter indicated that this gene is expressed mainly in the hypocotyl and affects leaf primordium development. These results suggest that the repression of *SPL13* by miR156 is essential for normal post-germinative growth in *Arabidopsis*.

Keywords: *Arabidopsis*, microRNA, miR156, post-germination, seedlings, *SPL13*

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Introduction

Seed germination and post-germinative growth are subject to transcriptional and post-translational control (Nonogaki *et al.*, 2007). For instance, genes associated with the biosynthesis and deactivation of plant hormones important for seed germination control, such as gibberellin (GA) and abscisic acid (ABA), are mainly regulated at the level of transcription (Yamaguchi *et al.*, 2007; Seo *et al.*, 2009). In contrast, GA and ABA signal transduction is mainly mediated by changes at the protein level. Components important for hormone signal transduction are subject to proteolysis or modification by the 26S proteasome pathway (Steber, 2007; Ariizumi *et al.*, 2008). While transcriptional and post-translational control of seed germination and seedling establishment

is well understood, the mechanisms concerning post-transcriptional regulation of genes associated with seed germination and seedling growth have not been fully investigated. Destabilization of mRNA, especially by microRNA (miRNA), has not been well characterized for seed germination and post-germination growth. miRNAs are expressed during seed imbibition and seedling growth (Martin *et al.*, 2005, 2006). Although a few reports have demonstrated physiological roles for miRNAs and miRNA target genes during seed germination and post-germination (Liu *et al.*, 2007), our understanding of miRNA function at these stages is still limited. In this study, we characterized the function of *SQUAMOSA PROMOTER-BINDING PROTEIN LIKE13* (*SPL13*) [see also the accompanying paper (Martin *et al.*, 2010) for general functions of *SPL*s in seeds and plants], which is targeted by miR156/miR157 (the sequences of miR156 and miR157 are nearly identical and the term 'miR156' will be used hereafter). The physiological roles of the *SPL13* and its regulation by miRNA at post-germinative stages are discussed.

Materials and methods

Generation of miR156-resistant SPL13 *mutants*

The *SPL13* gene (At5g50570) including the 1.3 kb upstream regulatory region was amplified from genomic DNA of *Arabidopsis thaliana* ecotype Col-0 using SPL13 forward (SPL13 F: 5′-ACCTACTCCTGCCAACACAATGTTCTTACA-3′) and reverse (SPL13 R: 5′-ATCCTACAAGATGGCTCATCTCAACAAGGT-3′) primers. The intact gene was used to generate non-mutated *SPL13* transgenic plants. Mutations at the miR156 target site were generated by site overlap extension polymerase chain reaction (PCR) mutagenesis (Ho *et al.*, 1989) using SPL13 mutant forward (SPL13mutF: 5′-CTGATTGTGCTCTCTCACTACTATCTTCCT-3′) and reverse (SPL13mutR: 5′-AGGAAGATAGTAGTGAGAGAGCACAATCAG-3′) primers. PCR products were cloned into pCAMBIA1301. *Arabidopsis thaliana* ecotype Col-0 plants were transformed by floral dip (Clough and Bent, 1998) using *Agrobacterium tumefaciens* carrying pCAMBIA1301 with intact *SPL13* (*SPL13*) or mutant *SPL13* (*mSPL13*) constructs. Wild-type and transgenic plants were grown at 22°C under 12-h light/12-h dark conditions until rosette stages, and then flowers were induced by transferring plants to 16-h light/8-h dark conditions.

mRNA and small RNA extraction

High molecular weight (HMW) RNA for mRNA expression analysis was extracted using a standard phenol–SDS extraction protocol. Briefly, 100 *Arabidopsis* seedlings were homogenized in 2 ml RNA extraction buffer [45.5% (v/v) phenol, 9% (v/v) chloroform, 0.45% (w/v) SDS, 41 mM LiCl, 2 mM EDTA, 5.9 mM β-mercaptoethanol, 82 mM Tris–HCl, pH 8.2] with a mortar and pestle. The extract was centrifuged at 10,000 *g* for 2 min. The supernatant was extracted with one volume of phenol–chloroform–isoamyl alcohol [25:24:1 (v/v/v)] solution and then with one volume of chloroform. LiCl was added to the supernatant (2 M final concentration) and the sample was mixed thoroughly and kept at −20°C overnight. The sample was thawed, mixed and centrifuged at 10,000 *g* for 5 min. The pellet was washed with 1 ml 80% (v/v) ethanol, dried, dissolved in water and used for mRNA expression analysis. To obtain low molecular weight (LMW) RNA for microRNA detection, the supernatant from the 2 M LiCl precipitation step in the total RNA isolation was fractionated by isopropanol (Martin *et al.*, 2005). The pellet from the 35–50% (v/v) isopropanol fraction was washed with 1 ml 80% (v/v) ethanol, dried, dissolved in water and used for miRNA expression analysis.

mRNA gel blot

An equal amount (2 μg) of HMW RNA was separated on a 1.3% (w/v) agarose gel containing 7% (v/v) formaldehyde, transferred to a positively charged Hybond-N + nylon membrane (GE Healthcare Bio-Sciences Corp./Amersham, Piscataway, New Jersey, USA), and UV-cross-linked. To make the RNA probe, the *SPL13* cDNA was cloned into the pCR-TOPO4.0 vector (Invitrogen, Carlsbad, California, USA) and transcribed using a digoxigenin (DIG)-labelled nucleoside triphosphate (NTP) mixture (Roche Applied Science, Indianapolis, Indiana, USA) and T7 RNA polymerase (Applied Biosystems/Ambion, Austin, Texas, USA). After a 15-min prehybridization at 60°C with a hybridization buffer containing 50% (v/v) deionized formamide, 4% (w/v) blocking reagent (Roche Applied Science), 0.2% (w/v) SDS, and 5 × SSC (sodium chloride/sodium citrate), hybridization was carried out in the same buffer containing approximately 100 ng ml^{-1} RNA probe for 16–18 h at 60°C. Membranes were washed once with 2 × SSC, 0.1% (w/v) SDS at 75°C for 25 min and then twice with 0.2 × SSC, 0.1% (w/v) SDS for 25 min at 75°C. Membranes were blocked for 30 min with 5% (w/v) non-fat milk in 0.1 M maleic acid buffer, pH 7.5, containing 0.15 M NaCl, and 0.3% (v/v) Tween 20 (buffer A) and were then incubated with alkaline phosphatase conjugated anti-DIG antibody for 1 h at 25°C. After washing with buffer A, the membranes

were subjected to chemiluminescence detection. The signal was detected on X-ray film after exposure.

Promoter analysis

The 5′ upstream region of *SPL13* (−1126 to + 185) was amplified using forward (SPL13-Pro-F: 5′-TCACCGA-GTATAGCCCAACAAG-3′) and reverse (SPL13-Pro-R: 5′-AAGTCGACACACCTACCAGTAGAGTC-3′) primers and cloned into the pCR-Blunt II-TOPO vector (Invitrogen). The region (−958/+185) was cloned into the *Sac* I and *Sal* I sites of a shuttle vector (Grebenok *et al.*, 1997) which contained the *uidA* (GUS: β-glucuronidase) gene. The promoter–GUS construct (4.4 kb) in pRJG23 was excised with *Sac* I and *Spe* I and subcloned into the *Sac* I and *Xba* I sites in the pGPTV-KAN binary vector (Becker *et al.*, 1992). GUS staining of seedlings was done as previously described (Weigel and Glazebrook, 2002) using 100 mM sodium phosphate buffer (pH 7.0) containing 0.1% (v/v) Triton X-100 and 2 mM X-Gluc (RPI Co., Mount Prospect, Ilinois, USA).

Results

Over-accumulation of miR156-resistant mutant form of SPL13 *(*mSPL13*)*

The miR156 target site (5′-GUGCUCUCUCUCUUCU-GUCA-3′) in *SPL13* mRNA contains one mismatched base and is located at a position corresponding to the amino acid sequence CALSLLS (Fig. 1A). *SPL13* was amplified from the *Arabidopsis* genome and four silent mutations were introduced at the miRNA target site (Fig. 1A). Constructs with the native promoter and the mutated *SPL13* (*mSPL13*) or intact gene (*SPL13*) were used to transform *Arabidopsis*. *mSPL13* plants over-accumulated *SPL13* mRNA, wild-type plants had low levels of *SPL13* and control transgenic plants with intact *SPL13* had intermediate levels of *SPL13* mRNA. An additional RNA band, which is most likely the product of RNA cleavage, was detected in control plants but was not detected in *mSPL13* (Fig. 1B). These results validated the deregulation of *mSPL13* mRNA from miR156.

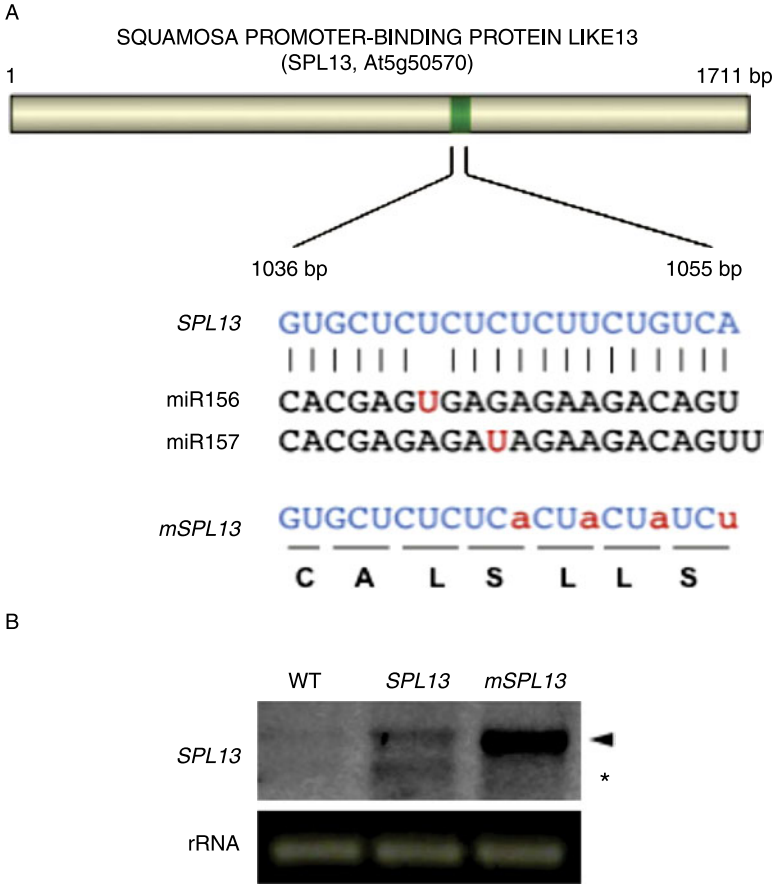

Figure 1. Over-accumulation of miRNA-resistant m*SPL13*. (A) The miR156 complementary sequence in *SPL13*. Silent mutations (no change in amino acid sequence CALSLLS) introduced into the miRNA-resistant form of *SPL13* (*mSPL13*) are in red. (B) RNA gel blot of *SPL13*. RNA was extracted 3 DAI from seedlings of wild-type (WT), transgenic *SPL13* and *mSPL13* plants. Ribosomal RNA (rRNA) bands are shown as loading control. Arrowhead and asterisk indicate the positions of intact and cleaved *SPL13* mRNA bands, respectively.

Post-germination phenotypes in the mSPL13 *mutants*

During phenotypic analysis, apparent differences were detected between control and *mSPL13* mutant plants following seed germination, while seed germination *per se* (radicle emergence) was not affected by the mutation. We found that many *mSPL13* seedlings (over 75%) had delayed transition from the two- to four-leaf stage (Fig. 2A). We stained normal and delayed seedlings from segregating populations of putative heterozygous mutant lines for transgene marker (*GUS*) (the transformation vector contained *35S:GUS* to identity transformants). The delayed seedlings with two vegetative leaves were GUS positive while the normal seedlings with four vegetative leaves were negative (Fig. 2B), indicating that the delay in seedling development was caused by the transgene, which caused over-accumulation of *mSPL13*. Three independent homozygous *mSPL13* transgenic lines were further characterized for this phenotype. All seedlings in the homozygous lines

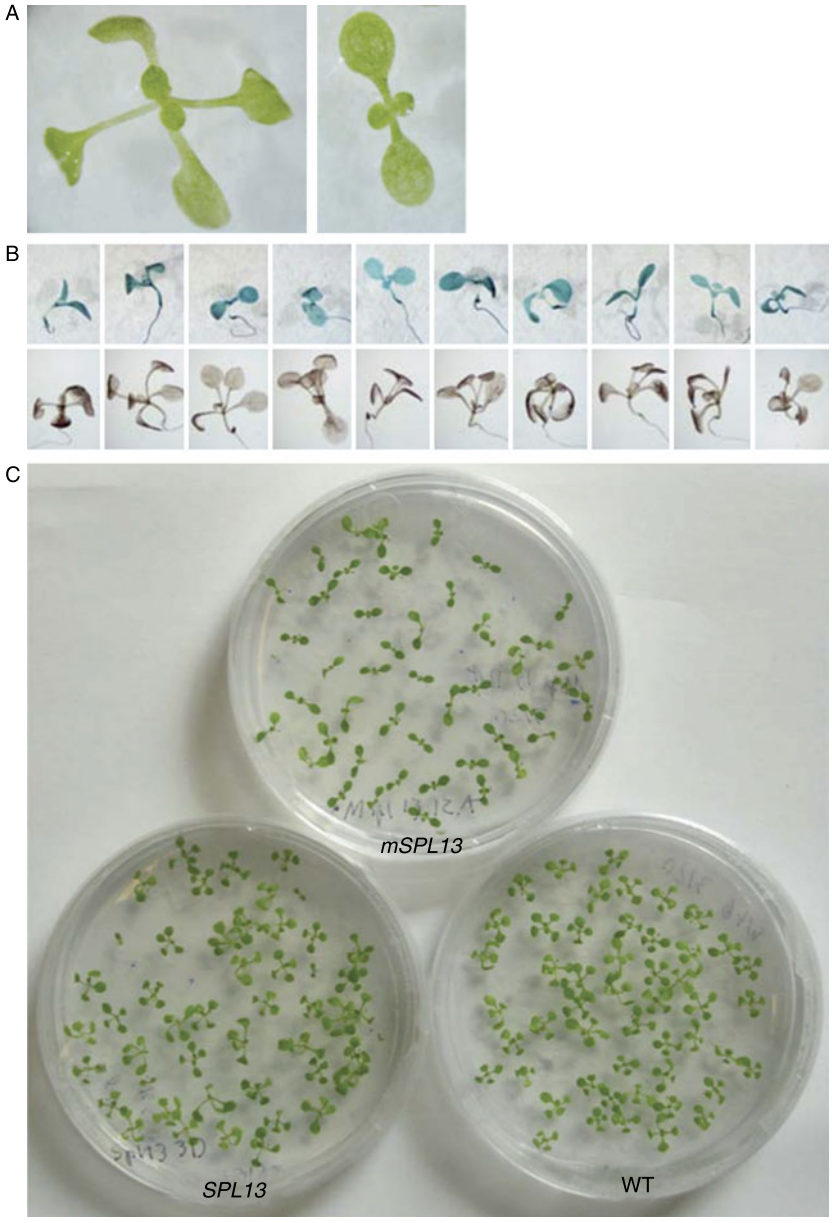

Figure 2. Delayed vegetative leaf emergence in *mSPL13* mutants at post-germinative stages. (A) A normal 10-DAI seedling with four vegetative leaves (left) and a delayed seedling with only two vegetative leaves found in putative heterozygous *mSPL13* lines. (B) GUS (β-glucuronidase, transgene marker) staining of the two- (upper row) and four-leaf seedlings (bottom row) from the heterozygous *mSPL13* population. (C) Comparison of 10-DAI seedlings of wild type (WT), control transgenic (*SPL13*) and homozygous *mSPL13* mutants (*mSPL13*).

exhibited delayed seedling development during post-germination stages. Wild-type seedlings and control transgenic seedlings expressing non-mutated *SPL13* reached the four-leaf stage 10 days after imbibition (DAI), while the *mSPL13* seedlings were still at the two-leaf stage after the same period (Fig. 2C).

Further analysis indicated that the difference between control and *mSPL13* mutant seedlings was initiated earlier, at the cotyledon seedling stages. The first two vegetative leaves emerged from the shoot apex of wild-type and *SPL13* seedlings 5–6 DAI (Fig. 3A). On days 4 and 5, leaf primordia were visible in the wild-type and *SPL13* shoot apical meristem (SAM) under a dissection microscope, while only a trace primordium was observed in *mSPL13* seedlings (Fig. 3B). Thus, the over-accumulation of *mSPL13*

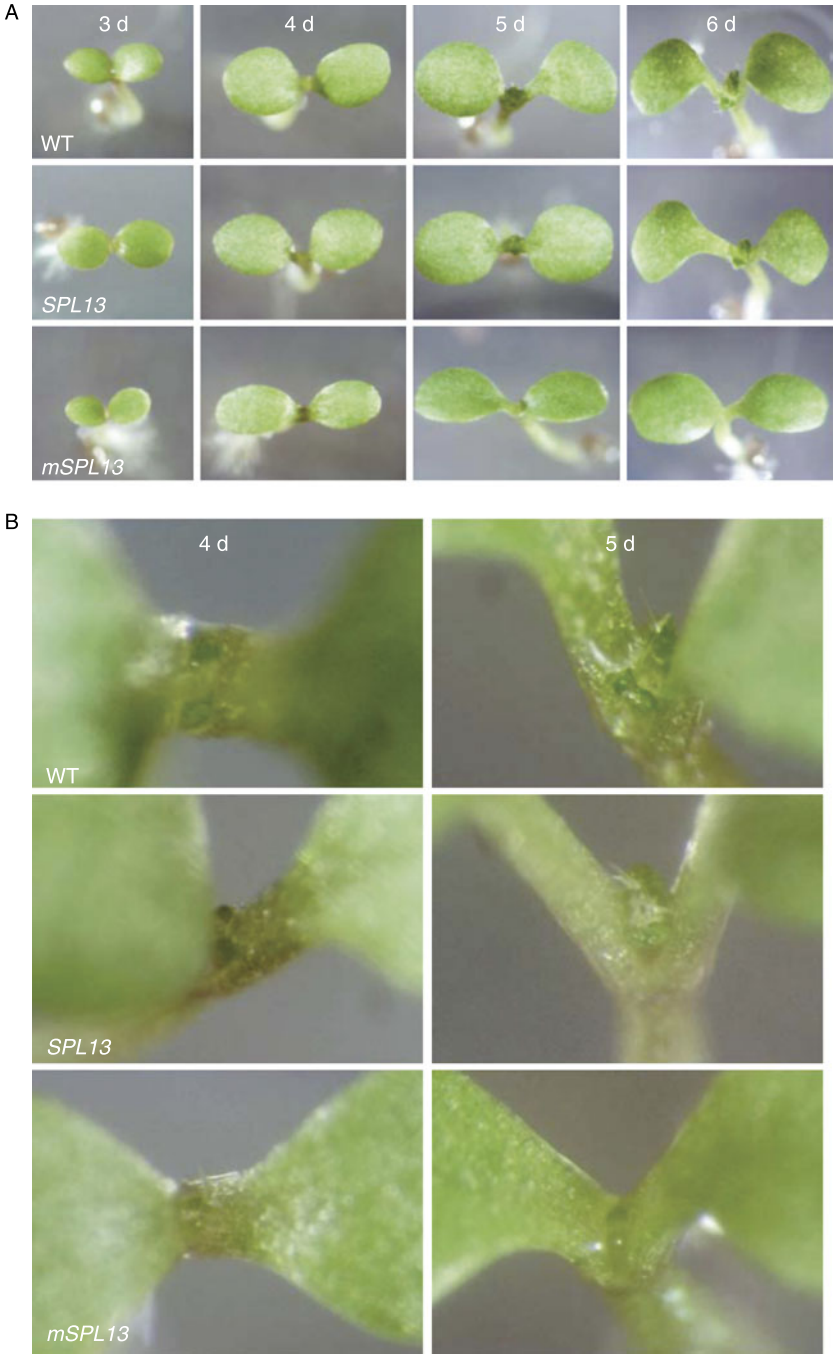

Figure 3. Delayed leaf primordium development in *mSPL13* seedlings. (A) Seedling development in wild-type (WT), control transgenic (*SPL13*) and mutant (*mSPL13*) at early stages (3–6 days). Note that there is a delay in vegetative leaf emergence in *mSPL13* seedlings. (B) Close-up view of the shoot apex in WT, *SPL13* and *mSPL13* seedlings.

suppressed the development of vegetative leaf primordia 3 DAI during the post-germinative stages.

Localization of SPL13 *expression*

The accumulation of *SPL13* in different plant developmental stages was analysed using the *Arabidopsis* eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). *SPL13* seems to accumulate in a broad range of tissues and organs during different developmental stages (data not shown). However, the accumulation of mRNA does not necessarily represent the expression of miRNA target genes, because miRNAs eliminate the target mRNAs by cleavage (except in the case of miRNA targets regulated by translational repression). In fact, *SPL13* mRNA does not accumulate at very high levels in seedlings, based on the analysis using the *Arabidopsis* eFP Browser (data not shown), suggesting that *SPL13* is normally suppressed by miR156 in seedlings. To see whether *SPL13* is actually expressed in cotyledon-stage seedlings, transgenic plants expressing GUS under the control of the *SPL13* promoter were generated and the expression of the reporter gene was analysed. Promoter analysis indicated that *SPL13* was expressed mainly in the hypocotyl of the seedling 3 DAI (Fig. 4). Weak signals were also detected in the vascular tissues at the basal part of cotyledons. These results indicate that *SPL13* is expressed in the hypocotyl of the cotyledon-stage seedlings, but is normally suppressed by miRNA156. The over-accumulation of miR156-resistant *mSPL13* in this tissue affected the emergence of vegetative leaves.

Discussion

The SQUAMOSA PROMOTER-BINDING PROTEIN (SBP) family is one of the plant-specific transcription factor families. *SBP* genes were first identified and characterized in *Antirrhinum majus* (Klein *et al.*, 1996) and were found to be widespread in green plants from unicellular algae (Kropat *et al.*, 2005) to angiosperms (Cardon *et al.*, 1999; Xie *et al.*, 2006). The SBP proteins regulate *SQUAMOSA* (*SQUA*) (Huijser *et al.*, 1992), a gene associated with floral induction in *A. majus*, by binding to a 32 bp (−295/−264) region in the 5′ upstream regulatory sequence of this gene (Cardon *et al.*, 1997). *SQUA* is an orthologue of *APETALA1* (*AP1*), a floral homeotic gene in *Arabidopsis thaliana* (Huijser *et al.*, 1992; Mandel *et al.*, 1992). Seventeen *SBP-LIKE* genes (*SPLs*) are found in the *Arabidopsis* genome (Cardon *et al.*, 1999). SPL3, SBP1 and SBP2 proteins interact *in vitro* with the *AP1* promoter element which has similarity to the SBP-binding motif in *SQUA*. The *AP1* and *SQUA* motifs contain 19-mer core sequences including an identical 10-bp conserved stretch (GTCCGTACAA) (Cardon *et al.*, 1997). Over-expression of *SPL3* causes early flowering (Cardon *et al.*, 1997) which is similar to the phenotype observed in the *AP1* over-expressors (Mandel and Yanofsky, 1995). This suggests that *Arabidopsis SPL3* modulates the timing of flowering through the regulation of *AP1*.

While *SPLs* were originally identified in relation to flowering mechanisms, they appear to play multiple roles in plant growth and development. Transposon mutagenized *spl8* plants exhibit reduced fertility primarily due to premeiotic abortion of the sporocyte, leading to abnormal development of microsporangia (Unte *et al.*, 2003). The *SPL8* gain-of-function mutants

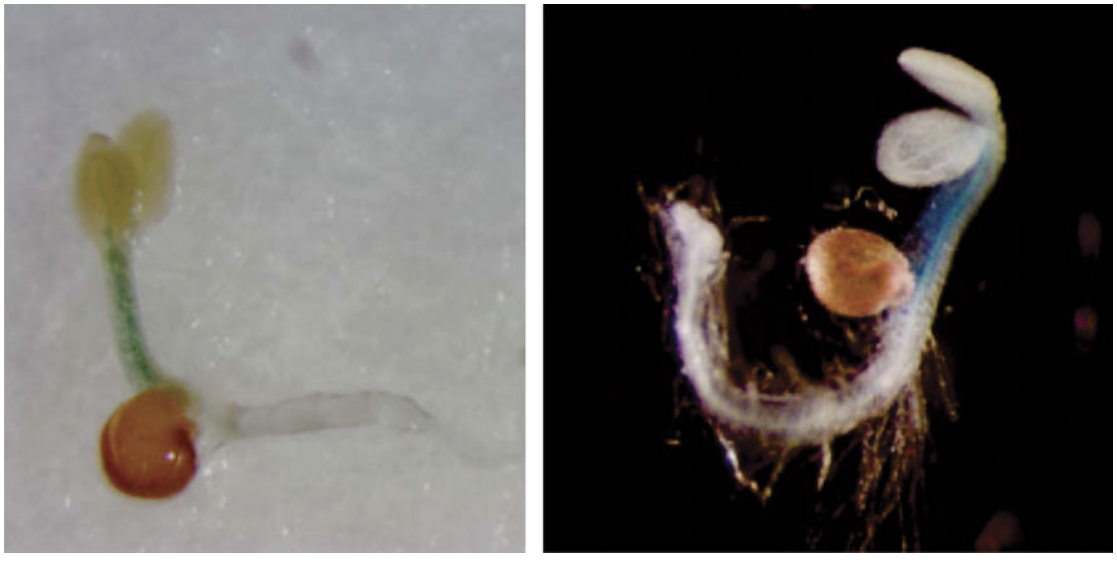

Figure 4. *SPL13* promoter:GUS expression in 3-DAI seedlings. The 5′ upstream region of *SPL13* (−958 to + 185) was used for the promoter analysis.

have nondehiscent anthers (Zhang *et al.*, 2006). These results suggest the involvement of *SPL8* in reproductive development. In addition, constitutive expression of *SPL8* resulted in dwarf plants exhibiting dark-green rosettes and compact inflorescences, phenotypes typical of gibberellin (GA)-deficient or GA-insensitive mutants. Seed germination, which is also regulated by GA, was reduced in seeds over-expressing *SPL8* (Zhang *et al.*, 2006), therefore *SPL8* may play a role in GA biosynthesis or signal transduction.

In the present study, deregulation of *SPL13* from miR156 did not cause phenotypes in seed germination *per se*. However, transgenic plants expressing the miR156-resistant *mSPL13* exhibited apparent phenotypes at post-germinative stages (Figs 2 and 3). As indicated by the promoter analysis, *SPL13* is actually expressed in the hypocotyl and in a part of the cotyledons (Fig. 4), but its mRNA does not seem to accumulate in these tissues, based on the output from the eFP browser analysis. These results support the contention that *SPL13* is negatively regulated by miR156 at the mRNA level. Consistently, silent mutations in the *SPL13* sequence complementary to the miR156 sequence caused over-accumulation of SPL13 mRNA, which caused a delay in seedling development. The function of *SPL13* is most likely to suppress the development of leaf primordia. This study demonstrates that post-transcriptional regulation of gene expression plays an important role at post-germinative stages in *Arabidopsis*. A possible mechanism to explain the consequence of *SPL13* over-accumulation is described in the accompanying paper (Martin *et al.*, 2010).

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